

The development or regeneration of plants containing the foreign, exogenous gene that encodes a polypeptide of interest introduced by *Agrobacterium* from leaf explants can be achieved by methods well known in the art such as described (Horsch *et al.*, 1985). In this procedure, transformants are cultured in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant strain being transformed as described (Fraley *et al.*, 1983).

This procedure typically produces shoots within two to four months and those shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Shoots that rooted in the presence of the selective agent to form plantlets are then transplanted to soil or other media to allow the production of roots. These procedures vary depending upon the particular plant strain employed, such variations being well known in the art.

Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants, as discussed before. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important, preferably inbred lines. Conversely, pollen from plants of those important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

A transgenic plant of this invention thus has an increased amount of a coding region (*e.g.*, a *cryIC** gene) that encodes the CryIC* polypeptide of interest. A preferred transgenic plant is an independent segregant and can transmit that gene and its activity to its progeny. A more preferred transgenic plant is homozygous for that gene, and transmits that gene to all of its offspring on sexual mating. Seed from a transgenic plant may be grown in the field or greenhouse, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for, by way of example, increased insecticidal capacity against lepidopteran insects, preferably in the field, under a range of environmental conditions. The inventors contemplate that the present invention will find particular utility in the creation of transgenic plants of commercial interest including

various turf grasses, wheat, corn, rice, barley, oats, a variety of ornamental plants and vegetables, as well as a number of nut- and fruit-bearing trees and plants.

4.12 METHODS FOR PRODUCING CRY1C* PROTEINS HAVING MULTIPLE MUTATIONS

5 Cry1C mutants containing substitutions in multiple loop regions may be constructed via a number of techniques. For instance, sequences of highly related genes can be readily shuffled using the PCR-based technique described by Stemmer (1994). Alternatively, if suitable restriction sites are available, the mutations of one *cry1C* gene may be combined with the mutations of a second *cry1C* gene by routine subcloning
10 methodologies. If a suitable restriction site is not available, one may be generated by oligonucleotide directed mutagenesis using any number of procedures known to those skilled in the art. Alternatively, splice-overlap extension PCR (Horton *et al.*, 1989) may be used to combine mutations in different loop regions of Cry1C. In this procedure, overlapping DNA fragments generated by the PCR and containing different mutations
15 within their unique sequences may be annealed and used as a template for amplification using flanking primers to generate a hybrid gene sequence. Finally, *cry1C* mutants may be combined by simply using one *cry1C* mutant as a template for oligonucleotide-directed mutagenesis using any number of protocols such as those described herein.

20 4.13 RIBOZYMES

Ribozymes are enzymatic RNA molecules which cleave particular mRNA species. In certain embodiments, the inventors contemplate the selection and utilization of ribozymes capable of cleaving the RNA segments of the present invention, and their use to reduce activity of target mRNAs in particular cell types or tissues.

25 Six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an
30 enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the

enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, 1992). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* (1992); examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. EP 0360257), Hampel and Tritz (1989), Hampel *et al.* (1990) and Cech *et al.* (U. S. Patent 5,631,359; an example of the hepatitis δ virus motif is described by Perrotta and Been (1992); an example of the RNaseP motif is described by Guerrier-Takada *et al.* (1983); Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990; Saville and Collins, 1991; Collins and Olive, 1993); and an example of the Group I intron is described by Cech *et al.* (U.S. Patent 4,987,071). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate